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(71) Applicant: GENENTECH, INC.
460 Point San Bruno Boulevard
South San Francisco California 94080(US)

(72) Inventor: Dull, Thomas Joseph
1850 Great Highway
San Fransisco California 94122(US)

(72) Inventor: Riedel, Heimo
480 Warren Drive No. 301
San Fransisco California 94131(US)

(72) Inventor: Ullrich, Axel
433 Upper Terrace
San Fransisco California 94117(US)

(74) Representative: Armitage, Ian Michael et al,
MEWBURN ELLIS & CO. 2/3 Cursitor Street
London EC4A 1BQ(GB)

(54) **Hybrid receptors, nucleic acid encoding them, their preparation, and their use in determination of ligands and their antagonists and agonists.**

(57) Hybrid receptors, produced by recombinant DNA technology, comprise (a) the ligand binding domain of a predetermined receptor and (b) a heterologous reporter polypeptide. The hybrid receptors are useful for convenient and large scale assay of biologically active ligands or their antagonists or agonists. (a) may be the extracellular domain of the receptor, or a cytoplasmic domain of a receptor or oncogene. (b) may be an enzyme. A transmembrane domain may be interposed between (a) and (b).

EP 0 244 221 A1

# HYBRID RECEPTORS, NUCLEIC ACID ENCODING THEM, THEIR PREPARATION, AND THEIR USE IN DETERMINATION OF LIGANDS AND THEIR ANTAGONISTS OR AGONISTS

This invention relates to methods for screening candidate drugs for their ability to bind a receptor in such a fashion as to mimic or antagonize the function of a ligand which ordinarily interacts with the receptor *in vivo*. It also relates to methods for the functional assay of ligands.

Receptors are defined as proteinaceous macromolecules located on cell membranes that perform a signal transducing function. Many receptors are located on the outer cell membrane. These cell surface receptors have extracellular and cytoplasmic domains wherein the extracellular domain is capable of specifically binding a substance so that the cytoplasmic domain interacts with another cell molecule as a function of the binding of the substance by the extracellular domain. The substance which is bound by the receptor is called a ligand, a term which is definitionally meaningful only in terms of its counterpart receptor. The term "ligand" does not imply any particular molecular size or other structural or compositional feature other than that the substance in question is capable of binding.

techniques do not lend themselves to large scale screening. Tissue samples or isolated cells containing the target receptors, e.g. heart atrial tissue, are costly to obtain, present in limited quantity, and difficult to maintain in a functionally viable state. Additionally, it is often difficult to reliably and reproducibly administer the candidate drug to tissue samples. Screening assays using primary explants in tissue culture are undertaken in larger scale than is possible with tissue samples. However, it is more difficult to assay physiological effect and the assays are subject to interference from many sources, e.g. culture media or cultivation conditions. Finally, assays using receptors isolated from natural materials have the disadvantage that the receptor is subject to natural variability and suitable natural sources may not always be available. It is an object herein to provide readily reproducible, simple assay systems that can be practiced on a large scale for determining not only ligand binding but also the character of the binding as agonistic or antagonistic.

Similarly, meaningful clinical diagnosis often depends upon the assay of biologically active ligand without interference from inactive forms of the ligand, for example, ligands that have been subject to enzymatic or other processes of the test subject that change or even eliminate the activity of the ligand. Immunoassay methods are widely used in determining ligands in test samples. However, it is often quite difficult to identify antibodies that are able to discriminate between the active and inactive forms of a ligand. Receptors have infrequently been used in place of antibodies as analyte binding reagents. However, not all substances that bind to receptors are necessarily capable of inducing receptor activity, i.e. active biologically. It is an object herein to provide a method that will identify ligands in clinical test samples which are active in inducing or inhibiting signal transduction by their receptors.

If a disease or injury is the result of a ligand acting on a given receptor, the objective will be to identify substances capable of counteracting the ligand's effect on the critical receptor, i.e., ligand antagonists. On the other hand, a model therapy for a clinical condition characterized by insufficient ligand activity would consist of drugs that enhance or supplement a defective or absent ligand, i.e., ligand agonists.

The hybrid receptor of this invention is useful in screening methods for identifying receptor-active agonistic drugs. One incubates the hybrid receptor with the candidate drug and assays for the generation of a signal by the heterologous reporter polypeptide. Generally, but not necessarily, the signal generated by the reporter polypeptide is assayed as an activation or stimulation of an enzymatic function of the reporter polypeptide. It is not necessary to include standards having known amounts of ligand unless one wishes to quantify the agonist activity of the candidate; in fact, the ligand which modulates the receptor activity in vivo may be completely unknown. It is one of the benefits of this assay system that neither the ligand for the receptor nor the in vivo signal transducing mechanism of the receptors need be known in order to identify agonist drugs.

The hybrid receptor is used to assay amounts of biological ligand in test samples in the same fashion as one screens for agonist drugs. Since this utility, by definition, contemplates a known ligand, then a standard curve using known amounts of ligand is prepared and compared with the test sample results.

Antagonist drug candidates are selected by the same assay as is used for identifying agonists, except that here the hybrid receptor is incubated with a known receptor agonist. The agonist, which may be a drug or the normal in vivo ligand, is

replication in E. coli.

Figure 1b is a schematic comparison of insulin (HIR) and EGF (HER) receptors and a hybrid receptors IER and IαER prepared therefrom. Human EGF receptor (HER), human insulin receptor (HIR), insulin-EGF receptor chimera (IER), and insulin-α-subunit-EGF receptor chimera (IαER) cDNAs are represented by horizontal lines and coding sequences shown as a dotted box for HIRα sequences (α), as a shaded box for HIRβ, and as an open box for HER sequences. The coding regions have been aligned at the transmembrane domain (not shown in scale). The coding segment for the protein signal sequence is marked by (S) and the precursor cleavage sites are indicated by a vertical line. The junction of the heterologous receptor cDNAs is shown by a zigzag line and synthetic oligonucleotides used at the junctions are represented by black bars. DNA restriction endonuclease cleavage sites relevant for the constructions are marked on top of the cDNA sequences.

Figure 2 illustrates that $^{125}I$ insulin binding to COS-7 cells increases when the COS-7 cells are transfected with the cDNA constructs of Figure 1a, compared to cells transfected with a control expression vector.

Figures 3a-3d are SDS PAGE reducing electrophoresis gels of autophosphorylated detergent lysates obtained from various transformed and control cells and immunoprecipitated with appropriate antibodies as noted in the Example. The (+) and (-) gels represent insulin or (in the case of A431) EGF-treated receptors. Numbers in the margins are marker molecular weights. Fig. 3a depicts the anti-HER immunoprecipitated autophosphorylation products of mock-transformed controls and recombinant transformant cells. This demonstrates expression of hybrid insulin-EGF receptor constructs in the recombinants.

tion.

Suitable ligand binding domains are selected in any one of several ways. First, when one intends to use the hybrid to assay for a known ligand in test samples, or to screen for agonists or antagonists to such ligand, then the ligand binding domain is selected from a known receptor for the ligand. If the ligand is known, but its receptor is not, then it will be necessary to identify its cell surface receptor. This may be accomplished by 1) securing cells from tissues with which the ligand is known to bind or to functionally interact, 2) obtaining from the cells in known fashion a membrane protein preparation, 3) incubating the preparation with the ligand, 4) separating the ligand-receptor complex from the incubation mixture (for example by preinsolubilizing the ligand on cyanogen bromide activated Sepharose), 5) separating the receptor from the ligand, 6) obtaining amino acid sequence from a portion of the receptor, 7) preparing nucleic acid probes encoding the determined amino acid sequence (either a single long probe of > about 40bp or a pool of shorter probes), 8) preparing a cDNA or genomic DNA phage or plasmid (vector) library from the organism or cells from which the receptor was obtained, 9) hybridizing the probes to the library to identify plasmids or phage which contain DNA encoding the receptor, and 10) determining the nucleotide and imputed amino acid sequence of the receptor to the extent necessary to identify the region extending from the amino terminus through a transmembrane sequence. If no single vector contains DNA encoding the entire extracellular domain of the receptor, the desired DNA is assembled by restriction enzyme digestion of the various vectors at common sites, isolation of the appropriate fragments and relegation by methods already known per se. Other procedures for identifying receptors for known ligands are known to those skilled in the art or will become available in the future.

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which is known to undergo a change in immunological or enzymatic identity upon ligand binding. It is preferred to use the cytoplasmic phosphotransferase from such receptors as the insulin or epidermal growth factor receptors. However, other receptors as the B-adrenergic receptor, acetylcholine receptor, adrenaline receptor and the like are known to bind proteins termed G proteins that serve as intermediate transducing molecules in the activation or inhibition of adenylate or granylate cyclases. Such proteins have been isolated and characterized. It is within the scope herein to use as the reporter polypeptide the G protein binding domains of such receptors. It is not necessary to use the entire cytoplasmic domain from a heterologous receptor or receptor analogue, only that portion that performs the desired function herein, nor is it necessary to use a heterologous cytoplasmic domain that is an intact, unmodified sequence from another receptor. For example, an amino acid sequence variant or derivative of the cytoplasmic domain of the receptor supplying the ligand binding domain is also acceptable.

Without being limited to a particular theory of function, we believe that the change in the character of the reporter polypeptide is not caused by steric hinderance of the reporter by the ligand, e.g. where the ligand occludes an active site on the reporter domain by virtue of steric bulk. Rather, the method herein harnesses the signal transducing mechanism of receptors whereby changes in the ligand binding domain are transduced through the receptor molecule to the reporter domain by conformational changes in the molecule, which changes affect the function or character of the cytoplasmic domain of the reporter. We have discovered that this transducing mechanism also functions when the reporter polypeptide is heterologous to the ligand binding domain.

Optionally, the hybrid receptor will contain a transmem-

the nucleic acid. The other function is to direct the expression of the hybrid receptor. One or both of these functions are performed by the vector-host system. The vectors will contain different components depending upon the function they are to perform as well as the host cell that is selected.

Each vector will contain nucleic acid that encodes the hybrid receptor. Typically, this will be DNA that encodes the hybrid receptor in its mature form linked at its amino terminus to a secretion signal. This secretion signal preferably is the signal presequence that normally directs the secretion of the receptor from which the ligand binding domain was obtained. However, suitable secretion signals also include signals from other receptors or from secreted polypeptides of the same or related species.

The secreted hybrid will lodge in the recombinant host membrane if it contains a transmembrane region. On the other hand, if such a region is not present in the hybrid, then the hybrid may be secreted into the culture medium. Ordinarily, hybrids are preferred that contain a transmembrane region so as to retain as much structural fidelity as possible. However, the purification of transmembrane-deleted receptors is less complex than in the case of membrane-bound because in the latter instance the hybrid receptor should be purified free of other cell membrane proteins. Furthermore, the cell-bound hybrid receptor may exert an undesired biological effect on the host if induced to accumulate in large populations in the cell membrane during the growth phase. This potential problem is overcome by placing the nucleic acid encoding the hybrid receptor under the control of an inducible promoter.

In cloning vectors, the hybrid receptor-encoding nucleic acid ordinarily is present together with a nucleic acid sequence

Tschemper et al., 1980, "Gene", 10: 157). The trp1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, 1977, "Genetics", 85: 12). The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, Leu2 deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the Leu2 gene.

Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase or proteins for neomycin resistance. Such markers enable the identification of cells which were competent to take up the hybrid receptor nucleic acid. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants in successive rounds of cell culture in which the concentration of selection agent in the medium is successively increased, thereby leading to amplification of both the selection gene and the DNA encoding the hybrid receptor. Increased quantities of hybrid receptor are synthesized from the amplified DNA.

For example, selection for DHFR transformed cells is conducted in a culture medium which lacks hypoxanthine, glycine, and thymidine. An appropriate host cell in this case is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, 1980, "Proc. Nat'l. Acad. Sci. USA" 77: 4216.

A particularly useful DHFR is a mutant DHFR that is highly resistant to methotrexate (MTX) (EP 117,060A). This

adaptors to supply any required restriction sites. Promoters for use in prokaryotic systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the hybrid receptor.

Suitable promoting sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (-Hitzeman et al., 1980, "J. Biol. Chem.", 255: 2073) or other glycolytic enzymes (Hess et al., 1968, "J. Adv. Enzyme Reg.", 7: 149; and Holland, 1978, "Biochemistry", 17: 4900), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triose-phosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned metallothionein and glyceraldehyde-3-phosphate dehydrogenase, as well as enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EP 73,657A.

Transcription from vectors in mammalian host cells is controlled by promoters and/or enhancers obtained from the genomes of bovine papilloma virus, vaccinia virus, polyoma virus, adenovirus 2, retroviruses, hepatitis-B virus and most preferably Simian Virus 40 (SV40), operably linked to the hybrid receptor nucleic acid. The early and late promoters of the SV40 virus are as conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication (Fiers et al., 1978, "Nature", 273: 113). Of course, promoters or enhancers from

filamentous fungi or yeast are suitable hosts for the hybrid receptor encoding vectors. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species and strains are commonly available and useful herein.

The preferred host cells for the expression of functional hybrid receptors are cultures of cells derived from multicellular organisms. In many cases, hybrid receptors contain hydrophobic regions that are incompatible with lower microorganisms, require complex processing to properly form disulfide bonds and often require subunit processing. In addition, it is desirable to glycosylate the receptors in a fashion similar to the native receptors. All of these functions can be best performed by higher eukaryotic cells. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture, although cells from mammals such as humans are preferred. Propagation of such cells in culture is per se well known. See Tissue Culture, Academic Press, Kruse and Patterson, editors (1973). Examples of useful mammalian host cell lines are VERO and HeLa cells, Chinese hamster ovary cell lines, and WI38, BHK, COS-7 and MDCK cell lines.

The hybrid receptors of this invention are employed in drug screening or biologically active ligand assay by a process that fundamentally comprises incubating the receptor with the test sample, controls and (optionally) standards, followed by measuring change in the reporter polypeptide. Since we have discovered that ligand binding causes a change in the conformation of the reporter polypeptide it is within the scope hereof to detect such changes by any one of several methods. Typically, one measures changes in the protein binding or enzymatic activity of the reporter polypeptide. In one embodiment an antibody is raised against the activated conformation and the binding of this

activity, primarily tyrosine kinase activity but in some cases serine or threonine kinase activity. Kinase activity is measurable in any way in which kinase activity has been assayed heretofore. One conventional, and presently preferred, method for kinase activity is to assay the incorporation of radiophosphorus into the reporter polypeptide through autophosphorylation with $^{32}P$. It is preferred to form hybrids of receptors having the same class of activity.

However, it is within the scope herein to measure changes in the reporter polypeptide by methods other than enzymological activity or polypeptide interactions. One such method contemplates binding an organic moiety to the receptor that undergoes a change in character upon ligand binding. For example, the reporter polypeptide is labelled with a stable free radical, a chemiluminescent group or a fluorescent molecule such as fluorescein isothiocyanate. Each of these labels are well known in the diagnostic immunochemistry art and conventional methods are well known for covalently linking them to proteins. These methods are useful for labelling the reporter polypeptide in the same fashion as other proteins. Changes in the conformation of the receptor polypeptide upon the binding of ligand or active candidate drug to the ligand binding domain are detected by changes in the label. For example, the rotational moment of a stable free radical label will be increased or decreased by ligand-activated changes in reporter polypeptide conformation. Similarly, the fluorescence or luminescence of reporter polypeptide labels will change upon the binding of ligand or active candidate to the receptor because of the reorientation of polypeptide species that engage in intramolecular energy transfers. This is detected by changes in the intensity, polarization or wave length of the label molecule; typically, one detects the enhancement or quenching of the label fluorescence or chemiluminescence. The advantage of the labelled reporter method is that

recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme infrequently is followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional (T. Maniatis et al., 1982, Molecular Cloning pp. 133-134).

"Filling" or "blunting" refers to the procedure by which the single stranded end in the cohesive terminus of a restriction enzyme-cleaved nucleic acid is converted to a double strand. This eliminates the cohesive terminus and forms a blunt end. This process is a versatile tool for converting a restriction cut end that may be cohesive with the ends created by only one or a few other restriction enzymes into a terminus compatible with any blunt-cutting restriction endonuclease or other filled cohesive terminus. Typically, blunting is accomplished by incubating 2-15μg of the target DNA in 10mM Mg $Cl_2$, 1mM dithiothreitol, 50mM NaCl, 10mM Tris (pH 7.5) buffer at about 37°C in the presence of 8 units of the Klenow fragment of DNA polymerase I and 250μM of each of the four deoxynucleoside triphosphates. The incubation generally is terminated after 30 min. by phenol and chloroform extraction and ethanol precipitation.

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the DNA from the gel. This procedure is known generally. For

## Example 1

## Construction of the Insulin Receptor (IR) Expression Plasmid

A gel purified SalI fragment (~5.2 kb) from λHIR-P12 containing the entire HIR coding sequence was subcloned into the pUC12 (New England Biolabs) polylinker region by digesting pUC12 with SalI and ligating the purified SalI fragment to the vector. Colonies were grown up and screened for clones having the desired orientation with the 5' end of the HIR coding sequence next to the pUC12 XbaI site. This vector was cut with XbaI and DraI (DraI is located in the 3' untranslated region of HIR) and the HIR-containing fragment was isolated. This fragment was inserted into a mammalian expression vector (pCVSVEHBVE400, European Publn. No. 117,060), which had been digested first with BamHI. The BamHI cohesive terminii were filled and the plasmid then was digested with XbaI. Thus, insertion of the XbaI-DraI was only possible in the orientation necessary for expression of the HIR mRNA. The resulting insulin receptor expression plasmid was designated pCVSV-HIRc.

## Example 2

## Construction of a Vector for Expression of an Insulin-EGF Receptor Hybrid

The following fragments were ligated in a four-factor ligation: (a) A 931 bp BamHI-AatII restriction fragment from the IR expression plasmid pCVSVE-HIRc, (b) a 1150 bp ApaI-SstI restriction fragment of the human EGF receptor sequence contained in the recombinant phage λHER-A64 (Ullrich et al., 1984, "Nature" 309: 418-425), (c) a synthetic oligonucleotide linker containing 5'-CCCGTCAAATATCGCCACTGGGATGGTGGGGGCC-3' and 5'-CCCACCATCCCAGTGGC GATATTTGACGGGACGT-3', and (d) pUC12 opened with SstI and BamHI.

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and cytoplasmic domains without any HIR B-chain sequence, was made by oligonucleotide-directed deletion mutagenesis of the IER plasmid. A 2.1 kb BglII restriction fragment coding for joined insulin and EGF receptor sequences was introduced into the BamHI site of an M13mp10 vector. Molecules with the desired orientation of the IR sequences next to the HindIII site of M13mp10 were identified and a single-stranded template was prepared for deletion mutagenesis with the oligonucleotide 5'-CCCCAGGCCATCTATCGCCACTGGGA-3' based on the protocols of Adelman et al. "DNA" 2: 183-193 (1983). 50 ng of phosphorylated primer was hybridized to 2 μg of single-stranded M13 template. The mutagenized second strand was completed and double-stranded molecules were introduced into E. coli JM101. Resulting plaques were screened as described by Benton and Davis "Science" 196: 180-182 (1977) at high stringency using the primer as a hybridization probe. Double-stranded DNA was prepared and a 1.2 kb BstEII restriction fragment containing the mutated region was used to replace the respective DNA fragment in the IER expression plasmid, yielding pIαER.

## Example 3

## Expression of the Hybrid Insulin and EGF Receptors

COS-7 monkey kidney cells (Gluzman, 1981, "Cell" 23: 175-182) were cultured in DMEM mixed with F12 medium (50:50), containing 10 percent fetal bovine serum and antibiotics. All cell culture media (Gibco) contained 2mM L-glutamine and 20mM HEPES pH 7.4.

pIER or pIαER from Example 2 was introduced into COS-7 cells by calcium phosphate coprecipitation based on the protocol of Graham and Van der Eb, 1973, "Virology" 52: 456-467. Subconfluent cells were transfected with 10 μg of plasmid DNA per 8 cm culture dish. Plasmid DNA was dissolved in 0.55 ml of 1mM

4°C for 5 min. The buffer which contained the solubilized cellular proteins was removed from the culture dish and centrifuged at 10,000g for 5 min at 4°C. Culture supernatants from transmembrane-deleted hybrid receptor transformed cells are centrifuged at 10,000 g for 5 min. at 4°C. 0.2 ml of the cell lysis or culture supernatant was incubated with 200nM insulin (Sigma) or 1 μM EGF for 1 hour.

A mouse monoclonal antibody capable of binding the insulin receptor (CII25.3, described by Ganguly et al., 1985, "Current Topics in Cellular Regulation" 27: 83-94) was insolubilized by adsorption to protein A-Sepharose. However, it will be appreciated that any polyclonal or monoclonal anti-insulin receptor antibody can be used. 1μl of antibody was mixed with 50 μl of a swollen and prewashed 1:1 protein A-Sepharose slurry in detergent-free lysis buffer for 30 min in order to adsorb the anti-IR antibody.

50 μl of insolubilized anti-IR antibody slurry was added to the EGF or insulin treated cell lysate or cell culture supernatant and incubated for 15 min. at 4°C. The resulting immunoprecipitate was washed 4 times with 0.9 ml HNTG buffer (20mM HEPES pH 7.5, 150mM NaCl, 10 percent glycerol, and 0.1 percent Triton X-100). The precipitate in a volume of 30 μl was adjusted to 5mM $MnCl_2$, and 15μCi of $\gamma$-$^{32}$P-ATP (5,000 Ci/mmol) was added for 0.5-10 min. at 4°C. The final ATP concentration was 0.1 pM ATP (for EGF, IER or IoER transformants and their controls) or 100 μM ATP (for HIR transformants and their controls). The autophosphorylation reaction was stopped by adding 20 μl of 3 times concentrated SDS sample buffer. The autophosphorylation reaction was terminated after 5 min. in Fig. 3a and 3d, 1 min. in Fig. 3b and after the times indicated in Fig. 3c by boiling for 5 min. The samples were centrifuged were and 20 μl aliquots analyzed on 5 percent/7 percent SDS polyacrylamide gels (Laemmli,

such as $Ca^{2}_{+}$/Calmodulin-dependent protein kinase (Kuret et al., 1985, "J. Biol. Chem." 260:6427-6433), type II cAMP-dependent protein kinase (Hemmings et al., 1981, "Eur. J. Biochem." 119:443-451).

Since uncleaved chimeric proreceptor IER displays insulin-stimulated autophosphorylation (Fig. 3b and 3c, IER + gel, top band), the tertiary structure necessary for insulin binding and signal transduction must be formed prior to insulin receptor proteolytic processing, consistent with previous reports (Blackshear et al., 1983, "FEBS" 158:243-246; Rees-Jones et al., 1983, "Biochem. Biophys. Res. Comm. 116:417-422). Our experiments with the chimeric construct IαER, in which the extracellular portion of the insulin receptor $\beta$ subunit and the proreceptor cleavage site are deleted, (Fig. 3b, IαER compare + and -) indicate that despite the apparent ability of the resulting 180 kd single-chain glycoprotein to bind insulin, insulin activation of the cytoplasmic kinase domain is lost.

As shown above, insulin regulates the rate of the EGF receptor autophosphorylation activity at subpicomolar concentrations of ATP, conditions under which the phospho-transferase of the insulin receptor is inactive. Hormone control was only observed for the hybrid IER containing the complete extracellular portion of the insulin receptor, including the signal for receptor processing into the $\alpha$ and $\beta$ subunits and the amino terminus of the $\beta$ subunit. The receptor appears to be processed in our expression system. In the case of the chimera IαER lacking any portions of the $\beta$ subunit and consequently the cleavage signal, no hormone effect was observed. We conclude that this structural difference between IER and IαER has a profound effect on the structure of the chimeric receptor that is crucial for signal transduction.

is ligated with the opened plasmid, the ligation mixture transfected into E. coli 294, and plasmid pCVSVE-HBS having the adaptor insert is recovered from an ampicillin resistant colony.

pCVSVE-HBS is partially digested with SacI and the linearized vector fragment (I) recovered. The linearized plasmid is digested with HpaI and the vector fragment recovered.

The pCVSVE-HBS vector fragment is ligated to the SacI-XmnI fragment encoding the hybrid receptor and expression vector pCVSV-HER-erbB was recovered from a transformed E. coli HB101 colony.

## Example 6

## Expression of Receptor-Oncogene Hybrid

Expression vector pCVSVE-HER-erbB is cotransfected into normal Rat 1 fibroblasts together with a neomycin resistable expression plasmid by calcium phosphate coprecipitation based on the protocol of Graham and van der Eb (1973). Subconfluent cells were transfected with 10 μg of plasmid DNA per 8 cm culture dish. DNA was dissolved in 0.55 ml of 1mM Tris pH 7.5, 0.1mM EDTA, 250mM $CaCl_2$ and 0.5 ml of 50mM HEPES pH 7.12, 280mM NaCl, 1.5mM $Na_2HPO_4$ was slowly added. A precipitate gradually formed within 40 min. which was added to the 10 ml of cell culture medium. 5h after transfection, cells were subjected to a glycerol shock treatment by incubation in 3 ml of 20 percent glycerol in PBS for 1 min. The glycerol was washed off and the cells were further cultured in the original medium.

The neomycin resistance gene under the control of the SV40 early promoter was used as a selectable marker. Medium supplemented with 400 μg/ml Geneticin (Sigma G5013) was used for

Example 7

EGF-stimulated in vitro autophosphorylation

To test whether HER-erbB possessed in vitro autophosphorylation activity, cell lysates were immunoprecipitated as described above, incubated with $^{32}$P-γ-ATP and analyzed by polyacrylamide gel electrophoresis and autoradiography: Transformant cell monolayers grown in 8 cm culture dishes were washed twice with PBS and solubilized as described by Kris et al. "Cell" 40:619-625 (1985). One ml of 50 mM HEPES pH 7.5, 150mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 10 percent glycerol, 1 percent Triton X-100, 1 percent Aprotinin and 4μg/ml phenylmethylsulfonyl fluoride (PMSF) was added to the monolayers at 4°C for 5 min. Solubilized cells were centrifuged at 10,000g for 5 min at 4°C, and the supernatant was either stored at -70°C or processed further.

EGF stimulation of autophosphorylation was induced by incubating the detergent cell lysates diluted to a 0.5 percent TX-100 concentration in 0.4 ml prior to the immunoprecipitation, with 5 μg/ml EGF for 15 min at 4°C. R1 antibody prebound for 30 min to protein A-Sepharose was added (1 μl antibody/50 μl slurry 1:1), and the incubation continued for 15 min at 4°C. The immunoprecipitates were washed 5 times in 0.9 ml HNTG buffer (20mM HEPES pH 7.5, 150mM NaCl, 10 percent glycerol, and 0.1 percent Triton X-100). The washed immunoprecipitates, in a volume of 30 μl, were adjusted to 5mM $MnCl_2$ and 15μCi of γ-$^{32}$P-ATP was added for 0.5 min at 4°C. The autophosphorylation reaction was stopped by adding 20 μl of 3 times concentrated SDS sample buffer. Samples were boiled for 5 min, centrifuged, and 20 μl aliquots analyzed on 5 percent/7 percent SDS polyacrylamide reducing gels (Laemmli, 1970). Gels were fixed and dried under vacuum at 70°C. Normal Ratl fibroblasts were used as a control. Size markers are indicated in kilodaltons. Like the wild type EGF receptor, the HER-erbB hybrid incorporated significant amounts of $^{32}$P in

CLAIMS

1. A hybrid receptor comprising (a) the ligand binding domain of a predetermined receptor and (b) a heterologous reporter polypeptide.

2. The hybrid receptor of claim 1 wherein the ligand binding domain is comprised by the extracellular domain of the predetermined receptor.

3. The hybrid receptor of claim 1 wherein the reporter polypeptide is a cytoplasmic domain of a receptor or oncogene.

4. The hybrid receptor of claim 1 wherein the reporter polypeptide is an enzyme.

5. The hybrid receptor of claim 4 wherein the enzyme is not sterically inhibited by the binding of a ligand to the hybrid receptor.

6. The hybrid receptor of claim 4 wherein the enzyme is a phosphorylkinase.

7. The hybrid receptor of claim 1 having a transmembrane domain interposed between the ligand binding domain and the heterologous reporter polypeptide.

8. Nucleic acid encoding a hybrid receptor comprising (a) the ligand binding domain of a predetermined receptor and (b) a heterologous reporter polypeptide.

9. The nucleic acid of claim 8 further comprising a replicable vector.

16. The method of claim 14 wherein the change in the reporter polypeptide is a modification of the enzymatic activity of the polypeptide.

17. The method of claim 16 wherein the change in the reporter polypeptide is autophosphorylation of the reporter polypeptide.

18. The method of claim 14 wherein the test sample is suspected to contain an antagonist and the receptor is incubated with the test sample and a predetermined activity of ligand or ligand agonist.

19. The method of claim 14 wherein the change in the reporter polypeptide is a change in an immune epitope.

20. The method of claim 19 wherein the change in immune epitope is detected by incubating the receptor with an antibody capable of binding to the reporter polypeptide and determining the amount of bound or residual unbound polypeptide.

21. The method of claim 14 wherein the reporter polypeptide further comprises a stable free radical, fluorescent or chemiluminescent group and the change in the reporter polypeptide is detected by measuring a change in the rotational moment of the stable free radical or a change in the intensity, wavelength or polarization of the fluorescent or chemiluminescent group.

22. The method of claim 14 wherein the reporter polypeptide is capable of binding to a G protein.

comprises a replicable vector.

10. The process of claim 9 which further comprises transforming a host cell with the vector.

11. A method for making a hybrid receptor which receptor comprises (1) the ligand binding domain of a predetermined receptor and (2) a heterologous reporter polypeptide, said method comprising:

(a) transforming a host cell with a vector containing nucleic acid encoding the hybrid receptor operably linked to a promoter for controlling the transcription of the hybrid receptor; and

(b) culturing the host cell under conditions for expressing the hybrid receptor.

12. The method of claim 11 wherein the hybrid receptor is recovered from the culture medium of the host cell.

13. The method of claim 11 wherein the hybrid receptor is recovered from the cell membrane of the host cell.

14. A method for assaying a biologically active ligand or an antagonist or agonist for said ligand, comprising:

(a) providing a hybrid receptor which comprises (1) a binding domain for the ligand, antagonist or agonist and (2) a heterologous reporter polypeptide;

(b) incubating the receptor with a test sample suspected to contain the ligand, antagonist or agonist;

(c) detecting a change in the reporter polypeptide; and

(d) correlating said change with the presence of the ligand, antagonist or agonist in the test sample.

15. The method of claim 14 wherein the ligand is a polypep-

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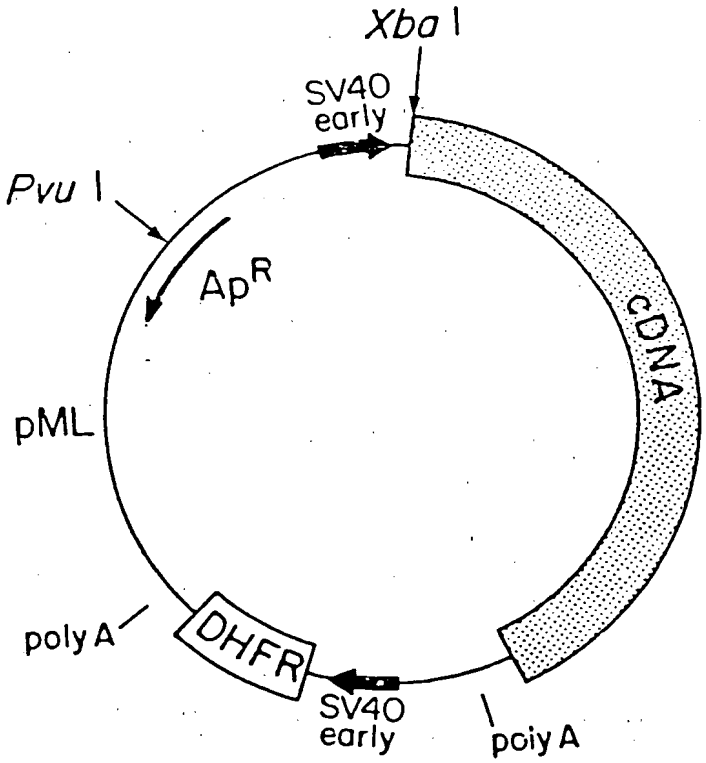
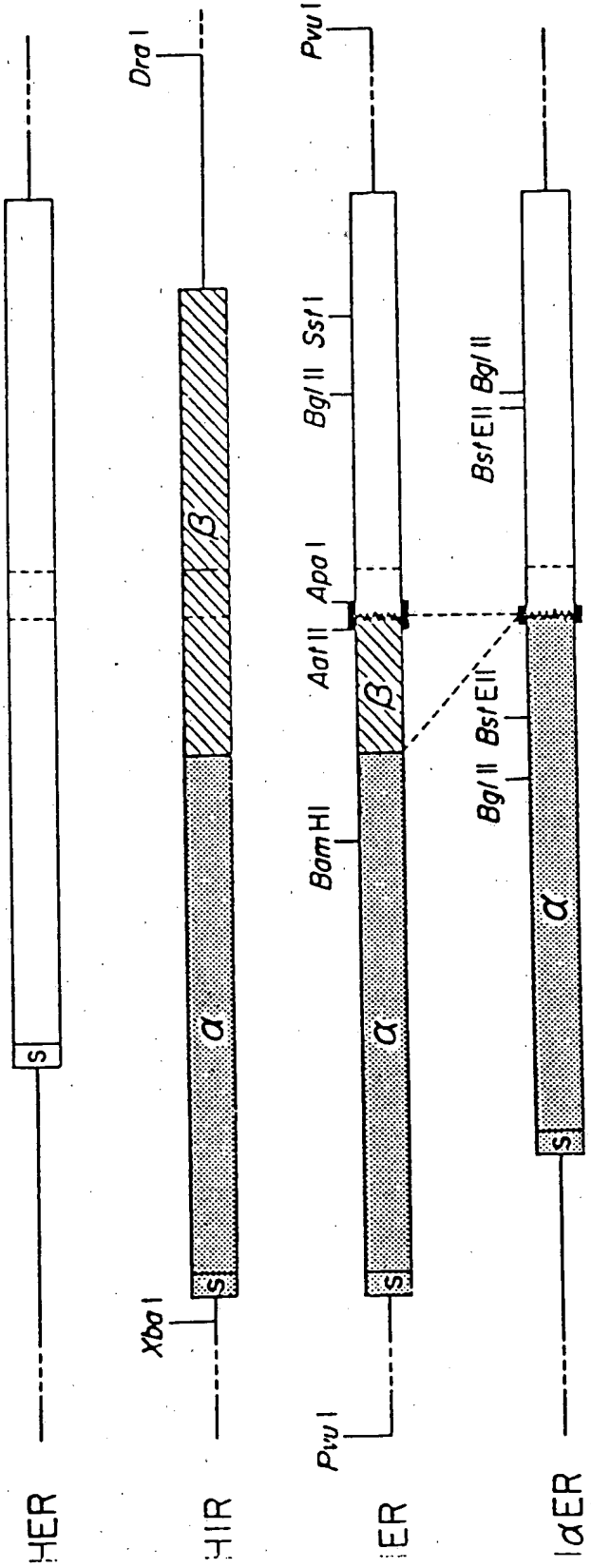
Fig.1a.




EXTRACELLULAR
TRANS-
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INTRACELLULAR
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HER
Dra I
β
α
S
Xba I
HIR
Pvu I
Bgl II Sst I
Apo I
Aat II
β
BamHI
α
S
Pvu I
IER
BstEII Bgl II
Bgl II BstEII
α
S
IαER

Fig.1b.

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Fig. 2.

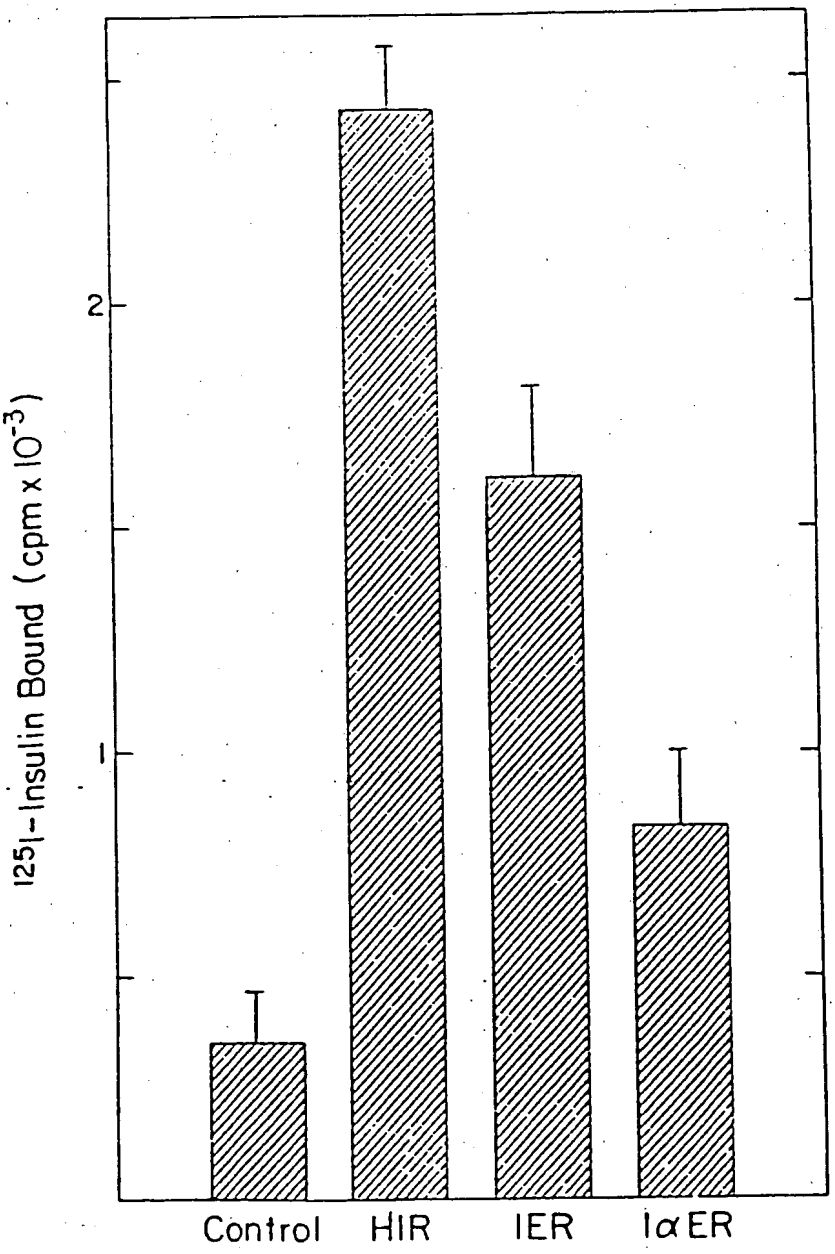


Fig.3.

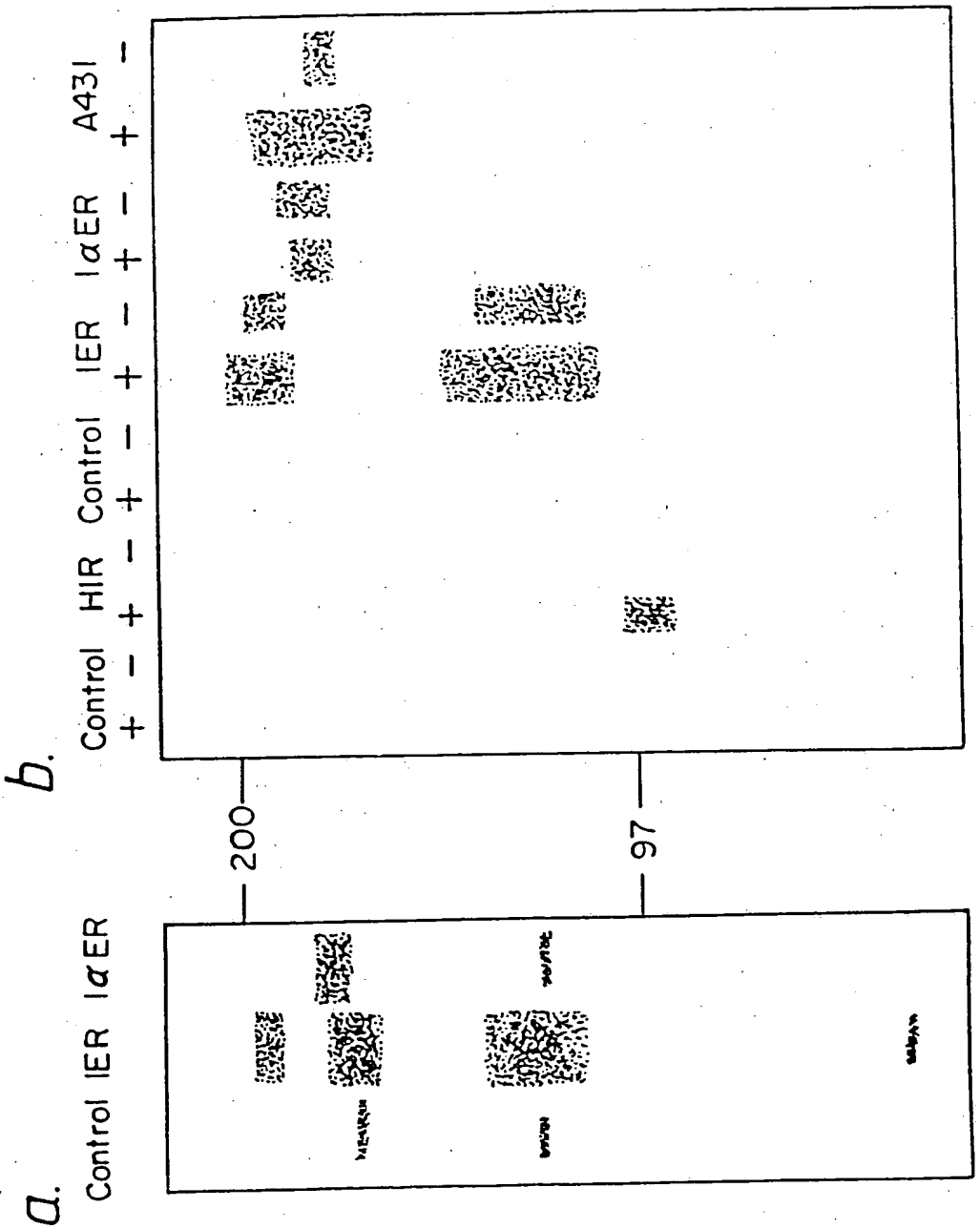


Fig.3.

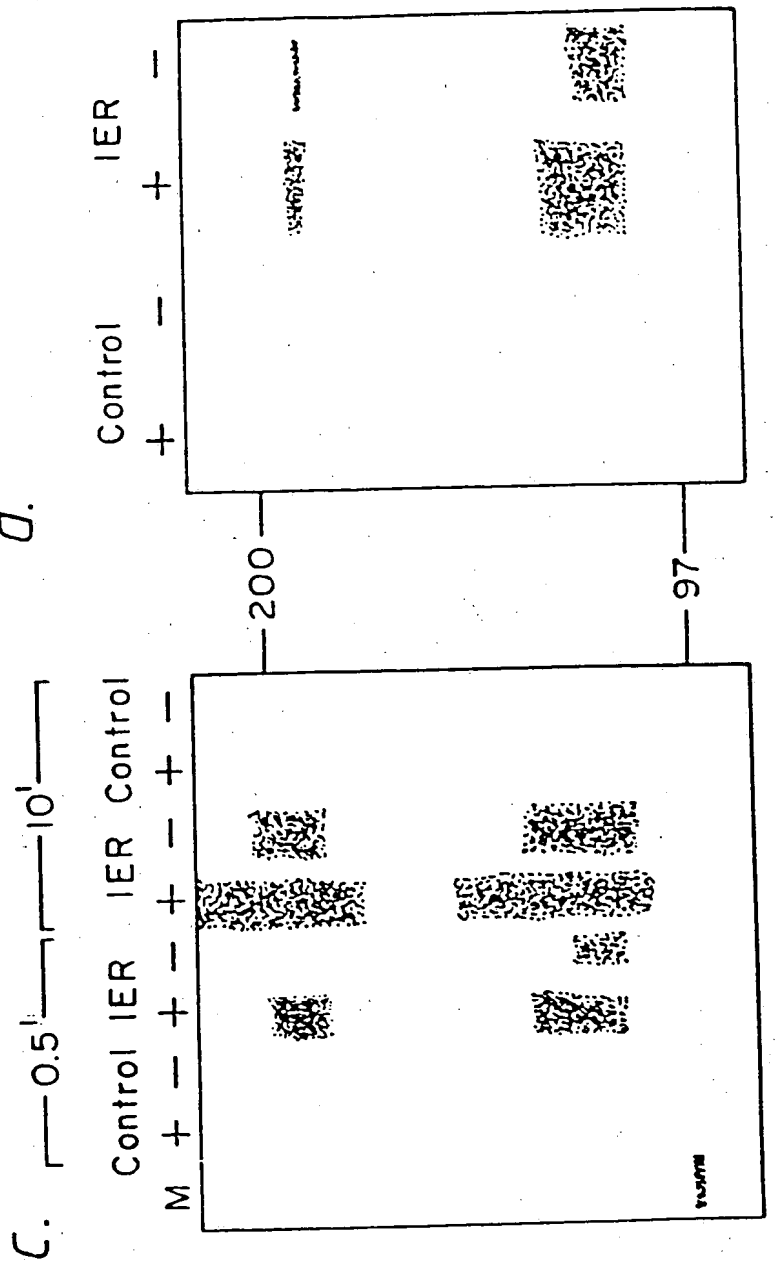
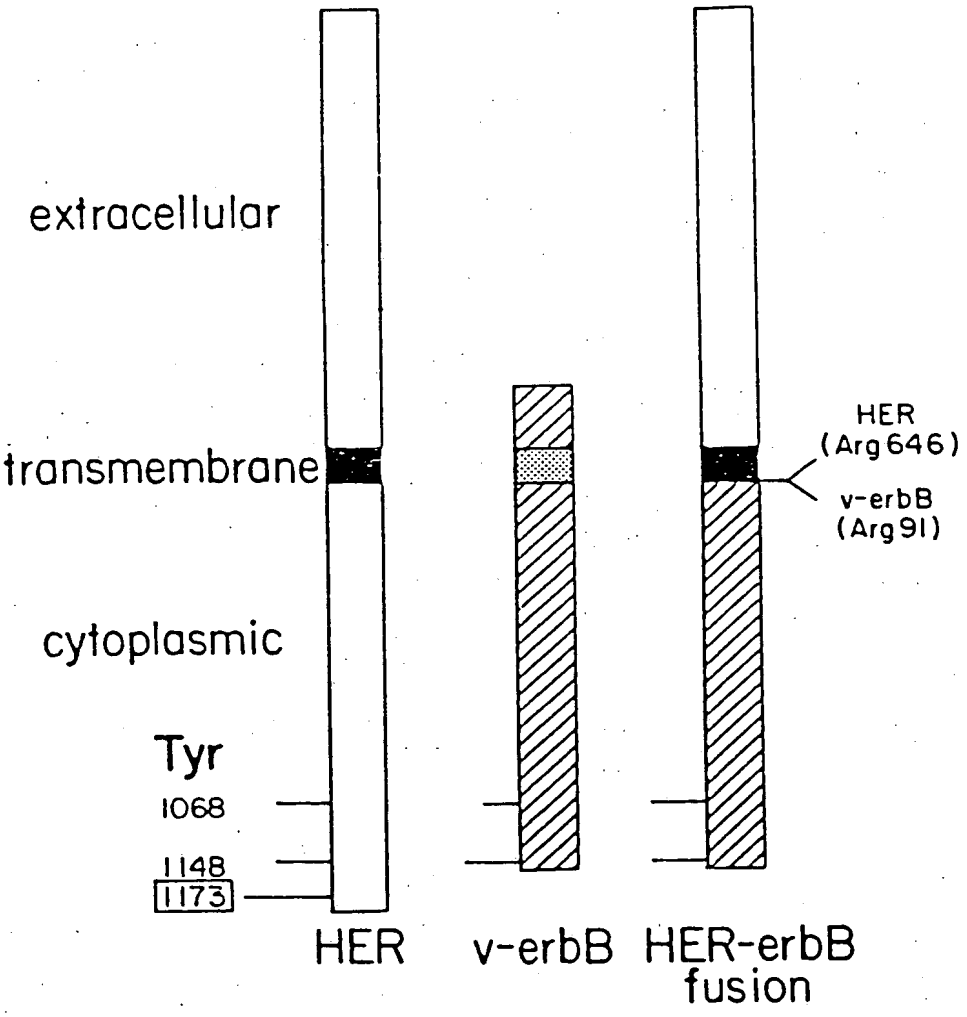


Fig.4.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cytoplasmic
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1148
1173
HER
v-erbB
HER-erbB
fusion
HER
(Arg646)
v-erbB
(Arg91)

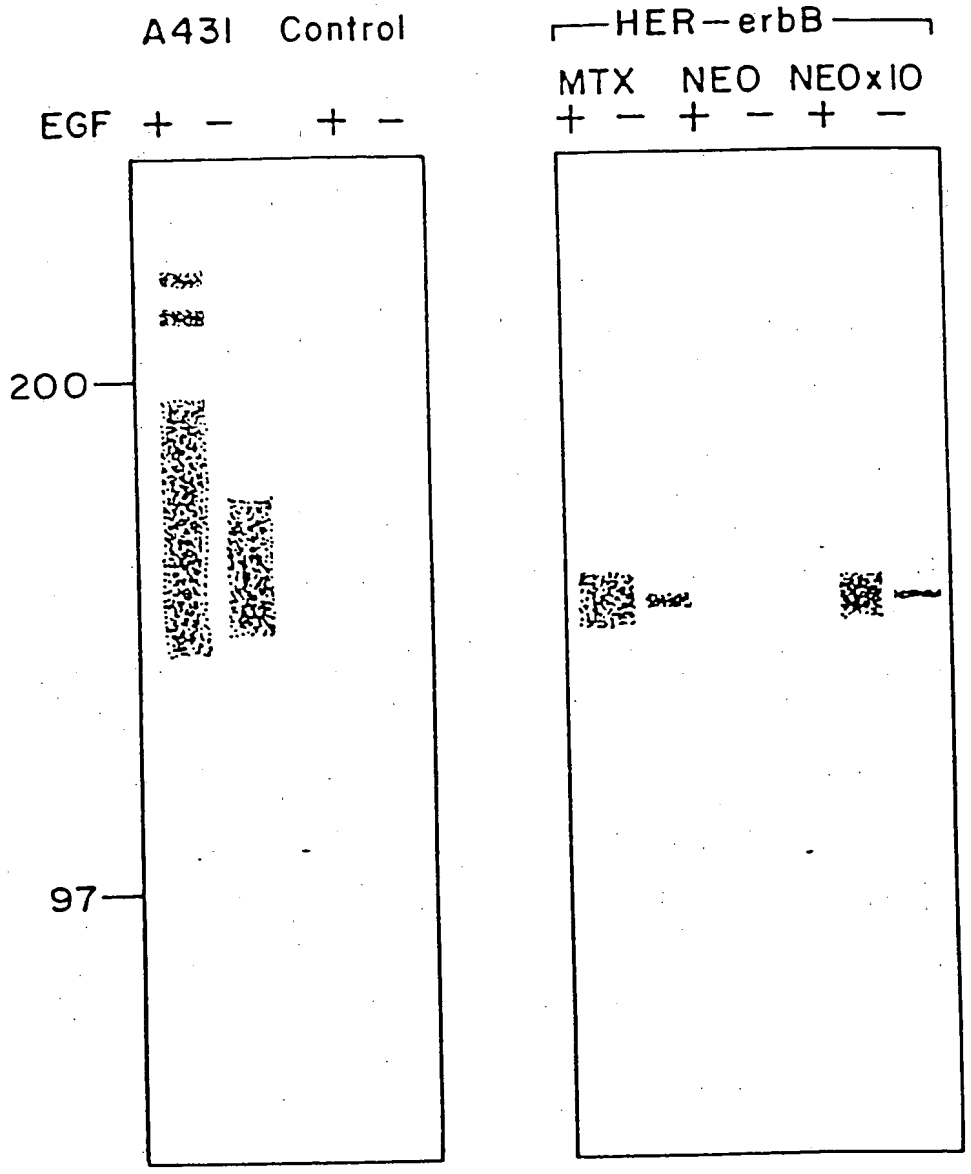


Fig.5.

European Patent Office

# EUROPEAN SEARCH REPORT

0244221
Application number

EP 87303801.2

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int Cl 4) |
|---|---|---|---|
| A | BIOCHIMICA ET BIOPHYSICA ACTA, vol. 738, 1984 (Amsterdam)<br>M. H. COBB et al. "The Insulin Receptor and Tyrosine Protein Kinase Activity"<br>pages 1-8<br>* Totality *<br>-- | 1 | C 07 K 15/00<br>C 12 N 15/00<br>C 07 H 21/00<br>G 01 N 33/53<br>G 01 N 33/68<br>C 12 P 21/00 |
| A | THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 256, no. 17, September 10, 1981 (Baltimore, USA)<br>T.W. SIEGEL et al. "Purification and Properties of the Human Placental Insulin Receptor"<br>pages 9266-9273<br>* Totality *<br>-- | 1 | |
| A | US - A - 4 504 587 (TIMPL et al.)<br>* Abstract *<br>-- | 14 | TECHNICAL FIELDS SEARCHED (Int. Cl.4)<br>C 07 K<br>C 12 N<br>G 01 N<br>C 12 P |
| A | DE - A1 - 3 100 061 (THOMA)<br>* Abstract *<br>---- | 14 | |

The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| VIENNA | 06-08-1987 | WOLF |

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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& : member of the same patent family, corresponding document

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A1

Office européen des brevets

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C 12 N 15/00, C 07 H 21/00
G 01 N 33/53, G 01 N 33/68
C 12 P 21/00

| No | références, formules, pages à photocopier, etc | No | classement |
|---|---|---|---|
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| 2 | | 2 | C12N15/20A |
| 3 | 0,37-42 | 3 | G01N33/532 |
| 4 | 0,37-42 | 4 | G01N33/566. |
| 5 | 0,37-42 | 5 | in/o G01N33/68 |

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